INTRODUCTION
AND
REVIEW OF LITERATURE
Concepts of a genetic type of therapy were developed almost 20 years ago and have recently been transformed into clinical reality. In 1990, the first gene therapy study for the treatment of ADA deficiency began (Blaese, et al., 1995), followed by a rapidly growing number of clinical gene therapy trials, across diseases caused by genetic disorders, viral infections and malignancies (Miller, 1992; Anderson, 1998). Current strategies are aimed at either the replacement of defective genes or suppression of a pathological gene target. At present, there are several well-documented approaches being pursued to affect the expression of defective gene production. A promising approach to decreasing the level of the abnormal protein(s) is represented by specific interference with gene expression at the level of mRNA. An emerging strategy in the treatment of viral infection is the use of antisense DNA or RNA to pair with and block the expression of viral transcripts.

The use of **antisense oligodeoxynucleotides (ODNs)** is an approach to study cellular and viral gene functions and to block gene expression in a therapeutic context. Antisense therapy mainly involves delivery into cells of small DNA oligonucleotides (7-30 nt in length), complementary to target RNA. These oligonucleotides specifically hybridize with the target RNA within the cell and interfere with the function of RNA by blocking RNA transport, splicing or translation. But, antisense therapy has its drawbacks in the sense that these are not enzyme molecules and hence have to be used in high concentrations, which might be toxic to the cell.

The finding that RNA can perform enzyme-like functions in cells overturned the view that biological catalysis is the exclusive realm of protein enzymes. The discovery of **ribozymes** by Cech and Altman (Cech, et al., 1981; Kruger, et al., 1982; Guerrier-Takada, et al., 1983) changed fundamentally our view of the function of RNA in chemistry, biology and medicine. RNA traditionally has been viewed as a passive molecule that only carries information
or provides structure. It is now clear that RNA can act as an enzyme, catalyzing not only RNA splicing and cleavage but also a diverse array of other reactions. The ability of catalytic RNA to facilitate chemical reactions previously associated only with protein enzymes has redefined the role RNA may have played in early evolution (Cech, 1993), and it may play in therapeutic approaches for the treatment of human diseases (Marschall, et al., 1994; Christofferson and Marr, 1995).

The notion that RNA has extensive catalytic properties is also consistent with the "RNA World" theory, which holds that ribozymes catalyzed a more varied metabolic chemistry prior to the invention of protein enzymes. In many ways, this theory has been the motivating force behind much of the effort to engineer ribozymes that perhaps represent the reinvention of past biological catalysts. In order for RNA to give rise to protein/DNA world of today, ribozymes alone would have had to catalyze a considerable number of chemical transformations, including reactions that are fundamentally different than phosphoester transfer and phosphoester hydrolysis reactions.

Kruger, Cech and coworkers (1982) first described RNA self-splicing in their studies of the 413-nucleotide group I intron from Tetrahymena thermophila. Few years later, Zaug and colleagues (1986) described a variant of the T. thermophila ribozyme, which could act in trans, i.e., as sequence specific endoribonuclease acting upon other RNA substrates. The work of Forster and Symons (1987b) concerning plant virusoid RNAs suggested that smaller RNA domains were capable of possessing ribozyme activity. Uhlenbeck (1987) was the first to demonstrate a small active ribozyme in which the catalytic and substrate strands were separated. Other simple ribozymes designed to act in trans were subsequently described (Koizumi, et al., 1988). The first published comments concerning the potential use of ribozymes as therapeutic agents appeared shortly thereafter (Haseloff and Gerlach, 1988).

The predominant activity associated with naturally occurring ribozymes is the ability to splice or cleave RNA molecules in a sequence specific
manner (for review, see Sun, et al., 2000). Sequence specificity results from the base pairing of ribozymes sequences with nucleotides around the cleavage site of the target RNA. Ribozymes can function in an intramolecular (cis) reaction to splice or cleave their own RNA sequence, and they can also function in a trans reaction to cleave another RNA molecule. Because of their sequence specificity, ribozymes show promise as therapeutic agents to downregulate a given RNA species within a background of cellular RNAs (Yu, et al., 1992; Sullenger and Cech, 1993; Flory, et al., 1996). Specifically, the mRNA encoding a protein associated with a disease state may be selectively cleaved. This cleavage event renders the mRNA untranslatable and attenuates expression of the protein product. In addition to naturally occurring ribozymes, the number of entirely synthetic RNA molecules with novel catalytic activities have been increased dramatically over the past few years as a result of in vitro selection and evolution techniques. Thus, the use of combinatorial methods is playing a central role in uncovering ribozyme functions that are likely untapped by modern cellular metabolism.

CLASSIFICATION OF RIBOZYMES:

There are several different classes of ribozymes: the self splicing group I and group II introns, RNase P and several different catalytic motifs found in the small pathogenic RNA which includes - hepatitis delta virus, hairpin ribozyme and hammerhead ribozymes.

GROUP I INTRONS:

Many eukaryotic genes have their coding sequences (exons) interrupted by stretches of noncoding DNA called introns. Transcripts of such genes must undergo cleavage-ligation reactions to produce the mature functional RNA. A large group of introns have been defined as "Group I" (Michel, et al., 1982) based on a set of conserved sequence elements, each about ten nucleotides in length (Michel, et al., 1982 and Waring, et al., 1982). The splicing of group I introns in the presence of a guanosine cofactor and magnesium was first observed for the intron of the nuclear 26S rRNA gene in *Tetrahymena*
thermophilica. Self-Splicing proceeds by two consecutive trans-esterification reactions, both initiated by nucleophilic attack. The excised intron, with a small deletion, can be converted into a true enzyme able to act in trans on specific substrates.

GROUP II INTRONS:

Group II introns have secondary structures and conserved sequences distinct from those of group I. These are commonly found in mitochondrial genes in plants, fungi, yeast and other lower eukaryotes. Like the group I introns, they are generally large fragments of RNA consisting of a series of helical domains and few single stranded regions of RNA. Self-splicing requires correct folding and the binding of requisite metal cofactors (Pyle, 1996). However, these differ with the group I introns in having no requirements for guanosine or any other free nucleotide as a substrate. The reaction requires spermidine, which presumably stabilizes the active conformation of the RNA. As with group I intron, the group II introns catalyze two sequential transesterification reactions that result in release of the intron and ligation of the flanking exon sequences.

RIBONUCLEASE P (RNase P):

Ribonuclease P (RNase P) is an ubiquitous ribonucleoprotein involved in processing the 5’ termini of tRNA precursors during their maturation. RNA cleavage is via nucleophilic attack on the phosphodiester bond leaving a 5’ phosphate and 3’ hydroxyl at the cleavage site, and there is an absolute requirement for divalent metal ions. The E. coli RNase P comprises of RNA domain of ~ 400 nucleotides (M1 subunit) and a protein component of 14 kDa dubbed C5. In vitro, the M1 RNA component has been shown to possess intrinsic catalytic activity; in vivo, however, there appears to be a requirement for the protein (Guerrier-Takada et al., 1983). The RNase P protein component is believed to facilitate binding between M1 RNA enzyme and tRNA substrate by masking electrostatic repulsion between enzyme and substrate RNAs (Reich, et al., 1988). RNase P can be directed to cleave any RNA when the target is in complex with a short, complementary oligonucleotide called an external guide.
sequence (EGS) that mimics the structure of a natural pre tRNA substrate, thereby inactivating it. A ribozyme (MIGS RNA) derived from the catalytic subunit of RNase P from E.coli has been shown to be highly effective in inhibiting replication of Herpes simplex virus 1 (Trang, et al., 2000).

**HEPATITIS DELTA VIRUS (HDV) RIBOZYME:**

The hepatitis delta virus (HDV) is a small single stranded RNA satellite of hepatitis B virus. Although it is a human pathogen, it shares a number of features with a subset of the small plant satellite RNA viruses, including self-cleaving sequences in the genomic and antigenomic sequences of viral RNA. The virus is thought to replicate via a double rolling-circle mechanism where both the positive and negative polarity strands promote self-cleavage (Fu and Taylor, 1993). The self-cleaving sequence is critical to viral replication and is thought to function as a ribozyme *in vivo* to process the products of rolling circle replication to unit length molecules. Several secondary structure models have been proposed for the HDV ribozyme and as described for the other ribozymes it is possible to design trans-acting constructs with the shortened forms (about 85 nucleotides in length) of this ribozyme as well (Thill, et al., 1993 and Lai, et al., 1996). The structural motif resembles neither the hammerhead nor the hairpin ribozymes. Many of the conserved nucleotides are located in helix II and its hairpin. The HDV ribozyme requires a divalent metal ion for activity and effectively uses both Ca$^{2+}$ and Mg$^{2+}$. Other metal ions like Mn$^{2+}$, Co$^{2+}$, Pb$^{2+}$ etc. also support cleavage but to a lesser extent (Suh, et al., 1993). A unique feature of the HDV ribozyme is its remarkable stability under denaturing conditions and high temperatures. Full cleavage activity is maintained in the presence of 20M formamide or 10M urea, and at temperatures up to 80°C, owing to the extremely stable secondary structure of this ribozyme (Smith, et al., 1992). The catalytic mechanism of HDV self-cleavage however utilizes the general acid-base catalysis as most of the known enzymes (Nakano, et al., 2000).
HAIRPIN RIBOZYME:

The hairpin ribozyme is derived from the self-cleaving domain of minus-strand satellite RNAs of tobacco ring spot virus (Hampel and Tritz, 1989 and Prody, et al., 1986). The hairpin ribozyme can function both in cis and in trans (Burke, 1994). Cleavage activity is dependent on divalent cations and gives rise to cleavage products containing 5'-hydroxyl and 2', 3'-cyclic phosphate termini. RNA cleavage by the hairpin requires the 2-amino group of the substrate guanosine immediately '3' of the cleavage site (Chowriria, et al., 1991). The shortened form of the hairpin ribozyme is also larger than the minimum hammerhead sequence. This bulky structure is the drawback of this motif since it increases the probability of many more secondary structures other than the main catalytic motif, which in turn leads to a decreased efficiency. However, di-ribozyme constructs of the hairpin have been made and they have been shown to cleave a suitable RNA substrate at two specific sites while maintaining the target specificity of the individual mono-ribozymes (Schmidt, et al., 2000). In a hairpin ribozyme, the catalytic RNA/substrate RNA complex forms a hairpin two-dimensional structure having four helical domains and five loop structures. Two helices form between the substrate and the ribozyme, which allow specificity of bonding. Located between these two helices in the substrate is the cleavage site, N*GUC where N is any nucleotide and cleavage occurs at position *. The maximal cleavage rate was obtained with the AGUC substrate. The hairpin ribozyme, like the hammerhead, effectively uses Mg$^{2+}$, Sr$^{2+}$ and Ca$^{2+}$ metal ions. However, unlike the hammerhead, it is inhibited by Mn$^{2+}$ and Co$^{2+}$ unless spermine is also present (Chowriria, et al., 1993). The hairpin ribozyme undergoes a single folding event induced by the binding of at least two metal ions, and involves close interaction between two internal loops to form the active ribozyme (Lilley, 1999). The hairpin ribozyme also catalyzes RNA ligation reaction that participates in processing intermediates of viral satellite RNA replication in plants. The hairpin ribozyme is a better ligase than it is a nuclease while the hammerhead ribozyme favors cleavage over ligation of bound products by nearly 200-fold (Fedor, 2000). Structural variants of the hairpin ribozymes are being
made and worked upon to optimize its catalytic performance (Barroso-delJesus, *et al.*, 1999).

**HAMMERHEAD RIBOZYME:**

Hammerhead ribozymes are small self-cleaving RNAs found in certain viruses and satellite RNAs that replicate via a rolling-circle mechanism (Turner, 1997 and Uhlenbeck, 1987). These were discovered initially in the self-cleaving domain of plus-strand satellite RNA of the tobacco ringspot virus (Forster and Symons, 1987a). The hammerhead is so called because of its secondary structure. Crystal structure of a naturally occurring hammerhead shows that it forms a compact V-shape, in which the catalytic center lies in a turn. The hammerhead structure has generated a lot of interest in its potential therapeutic applications since it is one of the best-characterized ribozymes. It is also very amenable to chemical synthesis because of its small and simple structure. The basic features of the hammerhead structure are the three base-paired stems I, II, and III, surrounding a single-stranded central region, with the 13 conserved bases (Ruffner, *et al.*, 1990 and Forster and Symons, 1987b). Stem I and stem III which are to the 5' and 3' of the catalytic domain respectively, form the hybridizing arms which base pair with the target RNA, whereas stem II is a stem-loop structure which is the main catalytic domain. The target cleavage site is a trinucleotide GUX* where X is A, U or C and cleavage occurs at position * resulting in formation of a terminus containing a 2', 3'-cyclic phosphodiester and a 5'-hydroxyl terminus on 3'-fragment. Perriman, *et al.*, (1992) further extended the target specificity to NUX where N can be A, U, G, or C. This states that any oligonucleotide with a NUX triplex can be cleaved by hammerhead ribozymes. Kinetic analysis revealed that GUC was cleaved most efficiently in a manner that depended both on $K_{cat}$ and $K_{cat}/K_m$ with CUC and UUC coming next (Shimayama, *et al.*, 1995). However, recently it has also been demonstrated that NAX or NCX triplets were also cleaved though at a rate much lower than that described for the GUC triplet. The target specificity of the hammerhead has thus now been reformulated to NXX from NUX (Kore, *et al.*, 1998). The hammerhead ribozyme is capable of cleaving in *cis* or in *trans* (Haseloff and Gerlach, 1988).
HAIRPIN RIBOZYME

Catalytic RNA

Substrate RNA

Site of cleavage

HAMMERHEAD RIBOZYME

5'-NNNNNNNUHNNNNNN-3' substrate

3'-NNNNNNNNA NNNNNN-5'

catalytic domain
In both configurations the ribozyme undergoes multiple turnovers and virtually any sequence can be targeted for cleavage. Mutagenesis studies have been carried out to identify the impact of nucleotide substitutions in the catalytic core (Ruffner, et al., 1990). Most of the substitutions led to a dramatic decrease in the cleavage activity. In addition, to the NXX triplets for cleavage specificity, the ribozyme arm sequence context can also influence cleavage rate significantly. In a simple term, the longer the binding arms the lower the turnover in cleavage of short substrates. The length of the 3' arm is apparently more critical for specificity than that of 5' arm (Hertel, et al., 1996).

The simplified catalytic cycle of a hammerhead ribozyme consists of:

- Sequence specific binding to the target RNA via complementary antisense sequence
- Site specific hydrolysis of the cleavable motif of the target strand
- Release of cleavable products.

The hammerhead ribozyme has an absolute requirement for divalent metal ions (Uhlenbeck, 1987). Mg\(^{2+}\) is the preferred metal, but Mn\(^{2+}\), Co\(^{2+}\), Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) also support cleavage activity (Dahm and Uhlenbeck, 1991). The hammerhead ribozyme undergoes a well-defined two-stage folding process induced by the sequential binding of two Mg\(^{2+}\) ions. The first corresponds to the formation of the ribozyme scaffold, while the second is the formation of the catalytic core of the ribozyme. Standard reaction conditions include 10mM of the metal ion, pH 7.5, at temperatures between 27\(^{0}\)C and 50\(^{0}\)C. In some cases additives that stabilize higher order nucleic acid structures, for example polycations spermine or spermidine, or sodium ions, can minimize the divalent metal ion requirement, but in most cases the divalent metal ion is still needed for complete activity (Dahm and Uhlenbeck, 1991). These observations imply that the metal ions may serve structural as well as catalytic roles. A single metal ion mechanism for coordinating the transition-state of a hypothesized catalytically active hammerhead ribozyme structure has been suggested (Murray and Scott, 2000).
SITE SPECIFIC CLEAVAGE OF RNA BY RIBOZYMES
ENGINEERED VARIANTS OF HAMMERHEAD RIBOZYMES:

To develop therapeutic ribozymes, significant research has been devoted to improve catalytic activity, develop nuclease resistance and optimize the intracellular delivery of both synthetic and expressed ribozymes. Many modifications have been engineered into the classical hammerhead structure, some of which are:

- **Asymmetric ribozymes**: These have a shorter stem I as compared to the conventional hammerhead ribozyme (McCall, et al., 1997; McCall, et al., 2000). Cleavage proceeds most rapidly when the length of the helix I is 5 base pairs.

- **Minizymes**: Minizymes are variants of hammerhead motif in which stem II has been replaced by a short linker sequence (Hendry, et al., 1995). The linker may be composed of ribonucleotides, deoxyribonucleotides or mixtures of these (McCall, et al., 1992), or non-nucleotidic moieties. Cleavage of gene length RNA substrates may be best achieved by miniribozymes (McCall, et al., 2000).

- **Maxizymes**: These are minizymes which form homo- or heterodimeric structures with two catalytic centers, two binding sites and a single, common stem II (Kuwabara, et al., 1996, 1999).

- **Allosteric ribozymes**: Ribozymes can function as allosteric enzymes that undergo a conformational change upon ligand binding to a site other than the active site (Soukup and Breaker, 1999). Hammerhead ribozymes that are activated by flavin mononucleotide (FMN) or theophylline have been engineered. A variety of other novel allosteric ribozymes have been made that function with other effector molecules, like cGMP, cAMP, cCMP (Koizumi, et al., 1999 and Marshall and Ellington, 1999). Allosteric hammerhead ribozymes have also been designed which are activated by the introduction of a short oligonucleotide (complementary to loop II) as a cofactor (Komatsu, et al., 2000).
Review of Literature

• Chimeric RNA-DNA hammerhead ribozyme: These are ribozymes in which the binding arms (stems I and III) were made of DNA (Hendry, et al., 1992). Rest of the ribozyme is made of RNA (Taylor, et al., 1992).

• Other chemical modifications - sugar, base and phosphate modifications, as well as complete substitution of natural nucleotides with non-natural linkers have been engineered in the ribozymes to improve the resistance to nucleases.

CATALYTIC MECHANISM OF RIBOZYME CLEAVAGE

Ribozymes are required to be correctly folded for activity. RNA folding might in principal have two results. First, the folded molecule might recruit metal ions into the correct position to participate directly in the chemistry of the cleavage reaction. Second, the RNA structure might itself function in the cleavage mechanism. The formal mechanism by which RNA cleavage occurs is still to be understood completely. Cleavage of RNA by ribozyme proceeds by Transesterification reaction. With the hammerhead, hairpin and hepatitis delta virus ribozymes, the 2'-hydroxyl adjacent to the scissile phosphodiester is employed as an internal nucleophile. This nucleophilic group attacks the phosphorus center to produce a pentacoordinate transition state or intermediate, which then breaks down to yield two fragments: a 2', 3'-cyclic phosphate product and a 5'-hydroxyl product.

Role of metal ions:

The role of metal ions in the chemistry of ribozyme cleavage is not fully understood and is rather a controversial subject. All known RNA catalysts have an absolute requirement for divalent metal ions, usually Mg$^{2+}$, but similar metals can often be substituted (Cech, 1987; Hunsicker and DeRose, 2000; Hanna and Doudna, 2000). Unlike most proteins, RNA requires the divalent metal ions for the formation of its three-dimensional structure. It is well known that metal cations bind to specific sites on an RNA sequence to stabilize specific structures. Therefore, catalytic RNAs utilize metals both for proper folding and for active site chemistry. Some metal ions can perform both functions, while
Review of Literature

others can either promote proper folding or participate in active-site chemistry. In the active-site chemistry, metal ions could deprotonate a nucleophile, activate an electrophile, stabilize a transition state, or protonate a leaving group (Yarus, 1993).

INTRACELLULAR EXPRESSION OF RIBOZYMES:
Successful use of ribozymes to knockout target gene expression is dependent on a number of factors, including target site selection as well as ribozyme gene delivery, expression, stability and intracellular localization.

Ribozyme Design: Not all target sites are accessible for cleavage; secondary structures, binding of proteins and nucleic acids, and additional esoteric factors influence in vivo ribozyme activity. Computer assisted RNA folding predictions and in vitro cleavage analysis are not necessarily predictive for in vivo activity, and the best Rz target sites often must be determined empirically in vivo. One strategy for enhancing activity is to design facilitator molecules: short RNA or DNA fragments complementary to the sequences flanking the Rz site. Binding of the facilitator can open up target secondary structure to allow Rz binding.

Ribozyme Delivery: There are two strategies to deliver ribozymes into cells: endogenous expression following DNA transfection and exogenous ribozyme transfection. Endogenous expression requires a vector – viral/plasmid containing the gene for the ribozyme under the control of a convenient promoter. Retroviral vectors are the most commonly used both in cell culture, primary cells, and in transgenic animals. Adenoviral vectors can be produced at high titers and provide very efficient transduction but they do not integrate into the host genome and therefore the expressions of transgenes is only transient in actively dividing cells (Huang, et al., 1997). Other viral delivery systems are actively being pursued such as the adeno-associated virus, alpha viruses and lentiviruses.

Based on experience from the use of antisense oligonucleotides, exogenous delivery of ribozymes is also been studied (Uhlmann and Peymann,
The major problem with the exogenous delivery system is that the ribozymes are exposed to serum containing culture medium, which is rich in RNases, resulting in their degradation (Taylor, et al., 1992). This process can be reduced by removal of the ribose 2' hydroxyl group from the ribozymes, which is required by RNases to cleave the phosphodiester bond. Synthetic ribozymes can also be stabilized by various base substitutions and modifications. Intracellular delivery in these cases can be achieved by lipid mediated transfection methods like liposomes and cationic lipids (Morgan and Anderson, 1993 and Castanotto, et al., 1994). Ribozyme gene constructs that are unresponsive to other methods of transfection can be propelled into primary cells using ballistic gene guns (Heiser, 1994).

Ribozyme Gene Expression: Stable intracellular expression of ribozymes could depend on the delivery system as well as the promoter choice which can allow temporal, constitutive or cell or tissue specific expression. A number of viral promoters using RNA polymerase II or III promoters, including tRNA, adenovirus VA1, U1 and U6 small nuclear RNA derivatives, have yielded high intracellular levels of ribozymes (Bertrand, et al., 1997). Furthermore, selectable markers (e.g. antibiotic resistance or surface molecules) can be co-expressed with the ribozyme to enforce high level expression. Intracellular localization of the Rz transcript is another important parameter. The U6 promoters preferentially confer nuclear localization, whereas tRNA-driven Rzs have been detected in nucleus and cytoplasm. In contrast, the adenovirus VA1 promoter targets the Rzs transcript specifically to the cytoplasm. Specific strategies to colocalize Rzs with their target RNA have also been developed to maximize intracellular Rz activity. Finally, as the field moves from cell culture to animal models, additional control over ribozyme expression will be required. Rz expression can be restricted to specific organs or cell types through the use of tissue specific promoters. This can be successfully done using the tyrosinase promoter, which is exclusively expressed in melanocytes. In a study of tissue specific expression, transgenic mice were made that carried a ribozyme gene driven by the insulin promoter. In the resulting adult mouse, ribozyme expression was limited to the pancreatic beta
Review of Literature

cells (Efrat, et al., 1994). Inducible promoters, such as those regulated by tetracycline, have shown utility in both cell culture and in animals, allowing ribozyme expression to be turned on and off at will (Juul, et al., 1997).

APPLICATIONS OF RIBOZYME:

The gradual maturation of ribozyme technology from the bench to clinical application involves major challenges. Ribozymes have been applied as anti-viral agents, treatments for cancer and genetic disorders and as tools for pathway elucidation and target validation.

Antiviral Therapy:

Initial uses of ribozymes focussed on anti-virals, primarily for the treatment of HIV, hepatitis B virus, hepatitis C virus. Several RNA viruses are known to have very high mutation rates, such as HIV and so immunization by conventional methods becomes difficult. Thus, use of ribozyme technology is an attractive alternative. Regions like promoter, splicing signal or packaging signal sequences in the viral genome are known to be more conserved. Targeting ribozymes to those sequences may magnify the effects against viral subtypes and reduce the emergence of escape mutants. Ribozyme against HIV have progressed into human clinical trials primarily through gene therapy approaches (Rossi, 2000; Paik, 1997; Chen, et al., 1992). Flossie Wong-Staal (Rowe, 1996) started the phase I clinical trials with a di-ribozyme (hairpin), encoded in a retroviral vector, against two highly conserved functionally important sequences of HIV-1. This vector was put into the peripheral blood cells taken from the patient, CD4+ T-cells expanded in culture, and then the T cells, now with the ribozyme genes inserted into the cellular DNA, were replaced in the patient. Flossie’s group (Wong-Staal, et al., 1998) has carried out another phase I clinical trial with autologous lymphocytes that have been transduced ex vivo with a retroviral vector bearing an HIV-1 leader sequence hairpin ribozyme. Preliminary results indicate that infusion of gene-altered, activated T cells in HIV infected patients is safe, and that transduced cells can persist for long intervals in HIV-infected subjects. Recently, a phase I clinical trial started with
the transduction of autologous CD34\(^+\) cells in HIV-1-infected patients with a Moloney murine leukemia virus (MoMLV)-based retroviral vector containing an anti-HIV-1 hammerhead ribozyme directed against the *tat* HIV-1 regulatory gene (Amado, *et al.*, 1999).

**Targeting cellular genes:**
Ribozymes have also been widely used to target cellular genes, including those aberrantly expressed in cancers. Ribozymes have been designed to specifically target bcr-abl fusion protein in chronic myelogenous leukemia (Wright, *et al.*, 1998). Rzs targeting overexpressed HER-2/neu in breast carcinoma cells effectively reduced their tumorigenicity in mice (Juhl, *et al.*, 1997).

**Against Genetic disorders:**
Heritable and spontaneous genetic disorders represent additional applications for therapeutic applications for targeting cellular genes. Ribozymes have been directed to target the beta-amyloid peptide precursor in an attempt to combat Alzheimers disease (Denman, *et al.*, 1997). Autosomal-dominant point mutations in the rhodopsin gene, which give rise to photoreceptor degeneration and the blinding disease retinitis pigmentosa, have been the target of ribozyme based therapy (Drenser, *et al.*, 1998). Chemically synthesized ribozymes directed against stromelysin mRNA have been injected into rabbit knee joints to reduce IL-1 induced arthritis (Flory, *et al.*, 1996).

**Anti-Cancer therapy:**
In addition to targeting oncogenes, Rzs have also been applied more directly as anti-cancer therapies. Ribozymes have successfully been targeted against a myriad of oncogenes including *ras* (Ohta, *et al.*, 1996), tumor necrosis factor-\(\alpha\) (Sioud, *et al.*, 1992 and MacKay, *et al.*, 1999) and *bcr-abl* (Lange, *et al.*, 1994). Ribozyme mediated inactivation of mutant K-ras oncogene in colon cancer cell line induced growth suppression, apoptosis and alteration of angiogenic factor expression. (Tokunaga, *et al.*, 2000; Tsuchida, *et al.*, 2000). An anti-p53 hammerhead ribozyme, designed to cleave the p53 mRNA, has been shown to effectively
Review of Literature

decrease the levels of endogenous mutant p53 mRNA (Cai, et al., 1995). Rzs targeting mdr-1 or fos could induce drug sensitivity of cancer cell lines (Funato, et al., 1997). Targeting bcl-2 with Rzs triggered apoptosis and increased phorbol ester sensitivity (Dorai, et al., 1997). Rzs targeting fibroblast growth factor binding protein (Czubayko, et al., 1997) and pleiotropin inhibited angiogenesis in mice, leading to decrease in tumor growth (Czubayko, et al., 1996). Many ribozyme-based anti cancer therapies could also be applied to other proliferative disorders, such as coronary artery restenosis.

Transgenic Research:
Transgenic animals are currently the only system for studying gene knockouts, both in the whole organism and in specific tissues. Ribozymes offer the equivalent of a gene knockout without the need for homologous recombination and inactivation of both alleles. Transgenic animals, containing a gene construct expressing either a complementary antisense RNA or a catalytic ribozyme sequence, have been suggested as potential models for developmental and tissue-specific downregulation of expression of a targeted gene in vivo which results in phenotypic consequences (Sokol and Murray, 1996 and Efrat, et al., 1994). Transgenic mice expressing Rzs directed against the glucokinase gene, under the control of the insulin promoter have been created. Glucokinase activity was specifically reduced by 70% in the pancreatic islet cells (Efrat, et al., 1994).

Pathway elucidation and target validation:
Rzs are unique in that they can inactivate specific gene expression, and thereby can be used to help identify the function of a protein or the role of a gene in a functional cascade. This application, target validation, is critical for both basic biological research and drug development. Introduction of ribozymes directed against matrix metalloproteinase-9 verified its role in sarcoma (Hua and Muschel, 1996) and prostate carcinoma (Sehgal, et al., 1998) metastasis. Ribozymes have been used as tools for therapeutic target validation in arthritis (Jarvis, et al., 2000).
Clinical applications of ribozymes:

The preclinical and clinical applications of ribozyme-based gene therapy has been primarily focussed on AIDS, cancers and other viral infections. This is summarised in table below.

<table>
<thead>
<tr>
<th>Company</th>
<th>Disease Target</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alza</td>
<td>CNS disease</td>
<td>Preclinical</td>
</tr>
<tr>
<td>American Cyanamid</td>
<td>Anti-ras ribozymes</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Columbia University</td>
<td>Anti- bcl-2 ribozymes</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Gene Shears</td>
<td>Anti HIV-1 ribozymes</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Immusol</td>
<td>Anti HIV ribozymes</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td>HBV, HCV, restenosis</td>
<td></td>
</tr>
<tr>
<td>Innovir</td>
<td>HBV, HCV, CML</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Osaka University</td>
<td>Anti-HCV ribozymes</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Ribozyme Pharmaceutical Inc</td>
<td>HIV-1 infection</td>
<td>Phase I/II</td>
</tr>
<tr>
<td></td>
<td>Angiogenesis</td>
<td></td>
</tr>
<tr>
<td>Tokyo University</td>
<td>HCV ribozyme</td>
<td>Preclinical</td>
</tr>
<tr>
<td>University of Pittsburgh</td>
<td>Glioma</td>
<td>Preclinical</td>
</tr>
<tr>
<td>City of Hope</td>
<td>HIV-1 infection</td>
<td>Phase I/II</td>
</tr>
</tbody>
</table>

In summary, a large body of work on ribozyme catalyzed efficiency, mode of action, and basic chemistry have accelerated the possibility of using ribozyme human gene therapy to target specific human diseases. With the development of delivery systems well under way, and with much deeper understanding of molecular genetics of human diseases, it is hoped that ribozymes will soon emerge as gene-targeted molecular therapies.
DNA-ENZYMES

RNA has well defined functions in cells, which span the range from the transfer of genetic information to molecular recognition and even biocatalysis. In stark contrast, DNA primarily serves a single purpose- the passive storage of genetic information. In nature, DNA exists primarily in its double stranded form. Within this compact helical conformation, DNA is considerably more resistant than RNA to degradation in aqueous solutions and hence is well suited to serve as reservoir for biological information. The distinctive biochemical properties of these two polymers have been ascribed exclusively to their differences in chemical composition. The presence of 2'- hydroxyl group, for example, renders the phosphodiester linkage of RNA much more susceptible to strand scission under biological conditions, compared with DNA. As a consequence, DNA is far superior to RNA as a format for long term storage of genetic information. Conversely, the 2'-hydroxyl group unquestionably contributes positively to the structural and functional complexity of RNA (Draper, 1996). Unlike the intricately folded structures of protein enzymes and ribozymes, continuous stretches of helical DNA are incapable of forming complex tertiary structures that are essential for a wide-ranging enzymatic functions. Outside the confines of the cells, DNA in its single stranded form can be made to perform both molecular recognition and catalysis- biochemical operations that were until recently thought to be possible only with macromolecules made of protein or RNA. For example, a number of DNA aptamers (Ellington and Szostak, 1990) can be made that function as ligands for proteins or as highly specific receptors for small organic molecules (Gold, et al., 1995; Breaker, 1997b). In addition, certain single stranded DNAs act as artificial enzymes (Breaker, 1997a), catalyzing such chemical reactions as phosphoester transfer (Breaker and Joyce, 1994,1995; Santoro and Joyce, 1997; Faulhammer and Famulok, 1997; Geyer and Sen, 1997), phosphoester formation (Cuenoud and Szostak, 1995), porphyrin metalation (Li and Sen, 1996), phosphoramidate cleavage (Carmi, et al., 1998), and DNA cleavage (Carmi, et al., 1996). The catalytic repertoire of DNA has greatly expanded in the recent years to include new substrates and new chemical
transformations. One of the most active areas of catalytic DNA research, however, has been the generation of *deoxyribozymes* that perform the chemical reaction - RNA cleavage by transesterification. Despite the recalcitrant character of double-stranded DNA and the paucity of chemical functional groups, early efforts to create deoxyribozymes from single stranded DNA using *in vitro* selection methods (Williams and Bartel, 1996; Lorsch and Szostak, 1996) have met with surprising success. Extensive search of DNA sequences was carried out seeking molecules that best met the following criteria:

1. Ability to cleave RNA with multiple turnover under simulated physiological conditions (example- 2mM MgCl₂/ 150mM KCl, pH 7.5, 37°C).
2. Ability to recognize the RNA substrate through Watson-Crick base pairing.
3. Generalizability to other RNA substrates by changing the sequence of the substrate recognition domain(s).
4. Catalytic efficiency meeting or exceeding that of comparable RNA enzymes.
5. Total composition of no more than 50 deoxynucleotide subunits.

**CLASSIFICATION OF DNA-ENZYMES**

**RNA - CLEAVING DEOXYRIBOZYMES:**
Distinct DNA motifs that require Mg²⁺, Pb²⁺, Zn²⁺, or Mn²⁺ as cofactors for RNA cleavage were isolated using a catalytic elution protocol. These DNA enzymes could be made to cleave almost any RNA substrate, efficiently and specifically under physiological conditions.

- **8-17 DNA-enzyme:** This DNA-enzyme recognizes 5'- AG -3' at the cleavage site and cleaves phosphodiester bond between A and G in the presence of Mg²⁺ ions (Santoro and Joyce, 1997). The catalytic core contained 13 deoxynucleotides and consists of a short internal stem-loop followed by an unpaired region of 4 -5 nt. The stem contains 3 bp at least 2 of which were G-C. The loop was invariant having sequence 5'-AGC-3'. Synthetic constructs in which the stem was lengthened or the sequence of the loop was altered did not exhibit catalytic activity. The 8-17 enzyme had a special requirement for a rG-dT “wobble” pair
located immediately downstream from the cleavage site. Substitution of a Watson-Crick pair at this position eliminated catalytic activity.

- **10-23 DNA-enzyme**: The 10-23 DNA-enzyme was named from its origin as the 23rd clone of the 10th cycle of *in vitro* selection (Santoro and Joyce, 1997). It has a catalytic domain of 15 deoxynucleotides, flanked by two substrate recognition domain of 7 to 8 deoxynucleotides each. The RNA substrate is bound through Watson and Crick base pairing and is cleaved at a particular phosphodiester bond between an unpaired purine and a paired pyrimidine residue. Target sites surrounded by A and U were cleaved most efficiently. The enzyme has very high kinetic efficiency rates approaching and even exceeding those of other nucleic acid and protein endoribonucleases. The catalytic efficiency ($K_{cat}/K_m$) of this Dz is $10^9 \text{M}^{-1}\cdot\text{min}^{-1}$, a value that is limited by the rate of RNA-DNA duplex formation. This remarkable activity is all the more spectacular when considering that it is achieved at concentrations of magnesium down in the physiological range. Experiments with different length binding domains have shown that the rate of Dz cleavage was highest in the molecule with a binding arm length ratio ($5'/3'\text{bp}$) of 6/10 (Cairns *et al.*, 2000a). These enzymes also have high substrate specificity and were able to discriminate between sequences that differ by as little as a single nucleotide (Cairns *et al.*, 2000b).

- **HD DNA enzyme**: The histidine dependent (HD) deoxyribozyme catalyses RNA transesterification in the presence of either L-Histidine or its corresponding methyl and benzyl esters (Roth and Breaker, 1998). This DNA-enzymes favor the cleavage of a single RNA linkage embedded within a DNA substrate with a rate enhancement of a million fold. It is proposed that histidine acts to deprotonate the 2' hydroxyl of the target linkage, thereby accelerating RNA transesterification. The histidine-dependent deoxyribozyme strongly discriminates against D-Histidine and a variety of histidine analogs, indicating that DNA is also capable of performing precise molecular recognition.
RNA CLEAVING DNA-ENZYMES

8 - 17 DNA-enzyme

![Diagram of 8-17 DNA-enzyme substrate and enzyme interaction]

10 - 23 DNA-enzyme

![Diagram of 10-23 DNA-enzyme substrate and enzyme interaction]

HD DNA-enzyme

![Diagram of HD DNA-enzyme substrate and enzyme interaction]
DNA-CLEAVING DEOXYRIBOZYMES:
Using \textit{in vitro} selection procedure two classes of DNA enzymes that self cleave using an oxidative mechanism were identified (Carmi, \textit{et al.}, 1996).

- **Class I self cleaving Deoxyribozyme:** requires both Cu$^{2+}$ and ascorbate for catalytic activity, which presumably serves as cofactors for the production of hydroxyl radicals.

- **Class II self cleaving Deoxyribozyme:** This DNA enzyme binds substrates using duplex and triplex recognition domains unlike most other RNA and DNA enzymes, which bind nucleic acid substrates exclusively by duplex formation. It requires presence of Cu$^{2+}$ ions for catalytic activity.

DEOXYRIBOZYMES WITH DNA - LIGASE ACTIVITY:
The “E47” DNA enzyme functions as a sequence- specific DNA ligase catalyzing the coupling of a 3’ phosphorimidazolye DNA substrate to the 5’- hydroxyl terminus of another DNA (Cuenoud and Szostak, 1995). Furthermore, E47 acts as a Zn$^{2+}$ or Cu$^{2+}$ dependent metalloenzyme, wherein the metal cofactor presumably performs both structural and catalytic roles (Sugimoto and Wakizaka, 1998).

DEOXYRIBOZYMES WITH PORPHYRIN METALATION ACTIVITY:
The PS5.M deoxyribozyme catalyses the insertion of Cu$^{2+}$ and Zn$^{2+}$ into a porphyrin substrate (Li and Sen, 1997). It is predicted to form a three tiered guanine- quartet structure that docks the porphyrin substrate in the gap that remains between the 5’ and 3’ termini. PS2.M, an 18-nucleotide variant of the metalase deoxyribozyme, (Travascio, \textit{et al.}, 1998) also displays weak peroxidase activity, although the mechanism for this action remains unclear.

DEOXYRIBOZYMES WITH KINASE ACTIVITY:
An individual, ATP- dependent, self- phosphorylating DNA enzyme ATP-2.1 was identified using \textit{in vitro} selection. This DNA enzyme forms a duplex and a three-tiered guanine quartet upon substrate binding. ATP- dependent phosphorylation occurs at the 5’ termini of the substrate oligonucleotide.

20
APPLICATIONS OF DNA-ENZYMES:
The ability of the 10-23 DNA-enzyme to specifically cleave RNA with high efficiency under simulated physiological conditions has fuelled expectations that this agent may have useful biological application in a gene inactivation strategy. Nuclease resistant DNA enzymes have been designed against protein kinase C- alpha (Sioud, et al., 2000). To explore the potential of DNA-enzymes, a number of groups have attempted to examine the activity of deoxyribozymes in biological systems. This is summarised in table below.

<table>
<thead>
<tr>
<th>Target</th>
<th>Cell</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-myc</td>
<td>SMC</td>
<td>80% suppression cell proliferation</td>
<td>Cairns et al., 1999</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>BV173</td>
<td>Apoptotic morphology</td>
<td>Warashina, et al., 1999</td>
</tr>
<tr>
<td>BCR-ABL-Luciferase</td>
<td>HeLa (transient)</td>
<td>99% suppression luciferase expression</td>
<td>Warashina, et al., 1999</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>K562</td>
<td>40% protein suppression, 50% cell proliferation</td>
<td>Wu, et al., 1999</td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>CD34+CML-bone marrow cells</td>
<td>53-80% suppression of growth in bcr-abl- positive CFU</td>
<td>Wu, et al., 1999</td>
</tr>
<tr>
<td>HIV-1 env</td>
<td>Hela (transient)</td>
<td>50% fusion</td>
<td>Dash, et al., 1998</td>
</tr>
<tr>
<td>CCR5</td>
<td>Hela (transient)</td>
<td>70% fusion</td>
<td>Goila and Banerjea, 1998</td>
</tr>
<tr>
<td>HIV-1env</td>
<td>U87</td>
<td>77-81% suppression viral load (p24)</td>
<td>Zhang, et al., 1999</td>
</tr>
<tr>
<td><em>huntington</em></td>
<td>HEK-293 (transient)</td>
<td>85% reduction in Huntington protein</td>
<td>Yen, et al., 1999</td>
</tr>
<tr>
<td>NGFI-A</td>
<td>SMC</td>
<td>75% proliferation</td>
<td>Santiago, et al., 1999</td>
</tr>
<tr>
<td>HIV-1 gag</td>
<td>Cos- 1</td>
<td>80% reduction</td>
<td>Sriram and Banerjea, 2000</td>
</tr>
</tbody>
</table>
HEPATITIS B VIRUS

The hepadnaviruses comprise a small group of enveloped DNA viruses. The most prominent members of this group are the human hepatitis B virus (HBV), the woodchuck hepatitis virus (WHV), the ground squirrel hepatitis virus, and the duck hepatitis virus. Although the recognition of hepatitis as an infectious disease dates to antiquity, the identification of hepatitis B virus (HBV) as one of its important causes was not achieved until the late 1960s. Within the past decade, however, the development of workable animal models of HBV infection, together with ongoing advances in molecular genetics, has brought these once-refractory areas into full experimental view.

THE BIOLOGY OF HBV INFECTION

Parental exposure of susceptible hosts to HBV results in primary infection of the liver. Although the classic result of such exposure is acute hepatitis B, a moderately severe illness characterized by hepatocellular injury and inflammation, many individuals experience mild or no liver injury despite extensive hepatic infection. Primary infection is usually self-limited, with clearance of viral antigens and infectivity from liver and blood and the development of lasting immunity to reinfection. However, 5-10% of the individuals do not resolve primary infection, but develop a persistent, usually lifelong, hepatic infection (Ganem, 1982). The unusual stability of infectious HBV virions, present mainly in the blood but also in other body fluids like saliva, urine and semen renders Hepatitis B highly contagious. Chronic HBV carriers, estimated to number over 300 million worldwide, represent the reservoir from which infection spreads to other susceptible individuals, either horizontally (chiefly via sexual contact) or vertically (from carrier mothers to newborn babies). Moreover, most of the mortality from HBV infection results from chronic rather than acute disease, severe chronic hepatitis B frequently leads to premature death from liver failure. But perhaps the most remarkable feature of chronic HBV infection is its association with the development of
primary hepatocellular carcinoma (HCC). The epidemiologic evidence linking chronic hepatitis B virus infection with the development of hepatocellular carcinoma is overwhelming: the incidence of HCC and the prevalence of HBV serological markers follow the same general geographical pattern of distribution, 80% of all HCCs occur in HBV infected individuals. In humans, 60-90% HCC patients have underlying cirrhosis, which is associated with the development of HCC in adenomatous foci that form within regeneration nodules of liver cells.

HEPATITIS B VIRAL GENOME

HBV is a small DNA virus with a partially double stranded 3.2kb genome (Robinson, et al., 1974). The viral genome consists of four defined open reading frames (ORFs) in the genome which result in transcription and expression of seven different hepatitis B proteins through use of varying in frame start codons (Bosch, et al., 1988, Ganem and Varmus, 1987, and Tiollais, et al., 1985). The four ORFs encode for the surface, core, polymerase and X proteins. There are many key domains other than the protein coding regions, which make this genome so unique. Interestingly, every base pair in the HBV genome is involved in encoding for at least one of the HBV protein but some areas also serve as regulatory regions, RNA transcription start sites, packaging signals and replication important domains.

Hepatitis B X Protein (HBx):

Hepatitis B virus has a unique fourth open reading frame coding for a 154 amino acid long protein known as Hepatitis B virus X protein (HBx). The HBx gene is well conserved among the mammalian hepadnaviruses and codes for a 16.5 kDa protein, which has been detected in both the nucleus and cytoplasm. The X gene contains its own promoter but requires the enhancer element for maximal activity (Treinin and Laub, 1987). In contrast to surface and core genes, the codon usage preference of the X gene is similar to that of eukaryotic genes (Miller and Robinson, 1986). HBx mRNA (0.7kb) has been
GENETIC ORGANISATION OF HBV GENOME
detected in infected liver, but the protein has not been easy to detect. However this protein must be expressed *in vivo* because antibodies against HBx have been detected in infected individuals (Kay, *et al.*, 1985; Meyers, *et al.*, 1986; Pfaff, *et al.*, 1987). HBx protein has been implicated in HBV mediated hepatocellular carcinoma (Tiollais, *et al.*, 1985; Robinson, 1994). Avian hepadnaviruses lack HBx (Rossner, 1992) which implies that HBx protein does not directly participate in genome replication or virion assembly. Upstream of this gene lies a region known as the HBV enhancer I which consists of several cis- acting sequences for efficient HBV expression. It appears that a portion of this enhancer region mediates the transcription of the HBV X gene. HBx protein is aptly named, as its complete function remains a mystery. A plethora of activities have been assigned to this protein.

**Properties and Functions of HBx**

- Several cellular genes, such as α1-antitrypsin and α-fetoprotein (Zhou, *et al.*, 1994) metallothionein (Zahm, 1988), epidermal growth factor (Menzo, *et al.*, 1993), MHC I (Zhou, *et al.*, 1990) and II (Hu, *et al.*, 1990), intracellular adhesion molecule I (Hu, *et al.*, 1992), β interferon (Twu and Schloemer, 1987) and interleukin-8 can also be activated by HBx.
HBx interacts with components of the basal transcription complex such as CREB/ATF2 (Qadri, et al., 1995; Williams and Andrisiani, 1995) and TATA binding protein (Qadri, et al., 1995), Oct 1 (Antunovic, 1993) and RBP5 subunit of RNA polymerase (Cheong, et al., 1995).

HBx stimulates cellular signaling pathway, Ras-Raf-MAP kinase cascade (Doria, et al., 1995), which leads to activation of transcription factors AP-1 and NFkB and enhanced proliferation in quiescent cells. It also activates Jak1-STAT signalling pathway in liver cells, which leads to proliferation of liver cells (Arbuthnot, et al., 2000). Thus, HBx acts as an "internal ligand" of the growth factor/cytokine-dependent signal transduction.

HBx can bind directly to tumor suppressor p53 protein (Wang, et al., 1994) and inhibit transactivation activity of p53 (Lee and Rho, 2000). HBx can sensitize p53-mediated apoptosis after exposure to DNA damaging agents and enhance apoptosis by tumor necrosis factor α (TNFα) and UV irradiation. HBx alleviates p53-mediated repression of alpha-fetoprotein expression (Ogden, et al., 2000).

The oncogenic potential of HBx is also suggested by observation of HCCs in HBx transgenic mice and oncogenic transformation of cells expressing HBx in culture which suggest that HBx may contribute to the pathogenesis of HBV-associated hepatocellular carcinoma (Kim, et al., 1991). Expression of HBx protein does not alter the accumulation of spontaneous mutations in transgenic mice (Madden, et al., 2000). Human HBx has also been shown to be a possible mediator of hypoxia-induced angiogenic hepatocarcinogenesis (Lee, et al., 2000).

HBx appears to be not only a substrate but also a potential inhibitor of the protease activities of cellular proteasomes (Zhang, et al., 2000). HBx can also bind to serine protease TL2 (Takada, et al., 1994), cellular DNA repair protein XAP-1/ UVDDDB (Becker, 1988) and simian virus large tumor antigen (Seto and Benedict, 1991).

HBx shows protein kinase C (Wu, et al., 1990) and ATPase activities (De-Medina, et al., 1994).
Mechanism of action of HBx

Several reports have suggested that HBx might stimulate transcription through direct interaction at the promoter with the components of the transcription machinery. On the other hand, the fact that HBx is not able to directly bind to DNA and that it activates a diverse group of transcription factors, including those of class III promoters, have been used to argue that HBx acts indirectly possibly by modifying the activities of cellular factors that modulate transcription (Aufiero and Schneider, 1990) through protein-protein interactions (Maguire, et al., 1991; Qadri, et al., 1995; Williams and Andrisani, 1995). Although there is no consensus among the regulatory regions of HBx-responsive genes, most of them incorporate cis-elements for some common trans factors like AP-1, AP-2, C/EBP, and NF-kB (Mahe, et al., 1991 and Seto, et al., 1990). Several studies have also indicated that HBx influences cellular signaling pathways (Lucito and Schneider, 1992; Cross, et al., 1993; Kekule, et al., 1993; Natoli, et al., 1994). HBx has been seen to inhibit TGF-β induced apoptosis by activation of phosphatidylinositol 3- kinase pathway (Shih, et al., 2000). Induction of apoptosis after switch on of HBx is mediated by the CRE/LOX recombination system (Shintani, et al., 1999). Other studies have proposed that HBx might act on the proteolytic degradation pathways of the cell (Takada, et al., 1994), including the proteasome complex. Furthermore it has been shown that X protein complexes with p53 tumor suppressor protein (Feitelson, et al., 1993; Truant, et al., 1995) and inhibits its sequence specific DNA-binding capacity, transcriptional activation function (Lee and Rho, 2000) and perhaps its indirect DNA repair function in vitro (Wang, et al., 1994). Some of the proposed activities of HBx are based on in vitro systems or interactions, making it difficult to determine which of these properties function within cells. Nevertheless, these studies clearly indicate that HBx is a multifunctional protein that displays a variety of independent activities, many of which could be important for virus infection or development of carcinoma.
Review of Literature

Human Immunodeficiency Virus (HIV)

Retroviruses are RNA-containing viruses that replicate through a DNA intermediate by virtue of a viral coded RNA-dependent DNA polymerase, also called reverse transcriptase (Fenner, 1975; Weiss, et al., 1984). Human Immunodeficiency Virus types 1 and 2 (HIV-1 and HIV-2) are members of the subfamily Lentivirinae of the family Retroviridae on the basis of genetic, morphological and pathological criteria (Gonda, et al., 1986; Haase, 1986). Both HIV-1 and HIV-2 can cause immune deficiency, but HIV-1 infection appears to be more virulent (Marlink, et al., 1994).

BIOLOGICAL PROPERTIES OF HIV

HIV replication is similar to that of other retroviruses and involves reverse transcription of the RNA viral genome to form a double-stranded DNA provirus. The early steps of replication lead to establishment of infection in target cells, but the virus may be dormant in some cell types, requiring activation for viral gene expression. One of the conspicuous features of HIV infection is a selective depletion of CD-4 bearing T-lymphocytes, suggesting a selective tropism and cytopathic effect of HIV for this population. The HIV-1 envelope glycoprotein gp120 binds to CD4 as indicated by their coprecipitation by antibodies directed against either of the proteins (McDougal, et al., 1986) This interaction leads to synctia formation and eventually cell death (Klatzmann, et al., 1984 a,b). Monocytes and macrophages have been shown to harbor HIV-1 in vivo and are likely to play an important role in the pathogenesis of the virus (Gartner, et al., 1986). CD4 helper T cells are not only quantitatively depleted but also are functionally abnormal in AIDS patient. Other cells of the immune system are also affected, directly or indirectly, leading to development of immunodeficiency, which in turn allows opportunistic infections of a variety of agents.
CHEMOKINES

Chemokines (a shortening of chemoattractant cytokines) represent a superfamily of about 30 chemotactic cytokines. They are small proteins (70-90 amino acid residues) with chemotactic activity for leukocytes; they play prominent roles in leukocyte activation and trafficking to sites for inflammation (Baggiolini, 1997). They are produced by wide variety of cell types. The production of chemokines is induced by exogenous irritants and endogenous mediators such as IL-1, TNF-α, PDGF, and IFN-γ. The chemokines bind to specific cell surface receptors (chemokine receptors) and exhibit more specialized functions in inflammation and repair. Receptors for chemokines (Murphy, 1996) comprise a subfamily within the G protein–coupled receptor superfamily. All known chemokines fit within four classes based on the cysteine motifs near the N-terminus. The two major classes are the CXC chemokines, in which the two cysteines are separated by a single residue, and the CC chemokines, in which the first two cysteines are adjacent. Nearly all the receptors are selective for one class of chemokines

Identification of HIV-1 Coreceptors:

Coreceptors for TCL-tropic HIV-1

The first HIV-1 coreceptor was identified using a functional cDNA cloning strategy based on the ability of a cDNA library to render a CD4-expressing murine cell permissive for fusion with cells expressing Env from T cell adapted strain (Feng, et al., 1996). A single cDNA was isolated and sequence analysis indicated that the protein product is a member of the superfamily of the seven transmembrane domain G-protein coupled receptors. Because of its newfound activity in HIV-1 Env-mediated fusion, the protein was named “fusin” (Feng, et al., 1996). Its role as a coreceptor was based on the demonstration that coexpression of fusin along with CD4 rendered non-human cells permissive for Env mediated fusion and infection. Also anti-fusin antibodies potently inhibited fusion and infection of primary human CD4+ T lymphocytes. Fusin thus fit the
criteria for T cell tropic HIV-1 coreceptor. It was later found that fusin is indeed a chemokine receptor specific for the functionally equivalent CXC chemokines SDF-1α and SDF-1β. Fusin was thus renamed \textit{CXCR4} (fourth receptor for CXC chemokines). SDF-1 was shown to be a selective inhibitor of T cell tropic HIV-1 strains.

\textbf{Coreceptors for M-Tropic HIV-1}

The discovery of fusin, a putative chemokine receptor, as the coreceptor for T-cell tropic HIV-1 strains provided a direction for identifying the coreceptor for M-tropic isolates. The focus was narrowed to CC chemokine receptors. The first success was achieved in 1995 with the demonstration that the CC chemokines RANTES, MIP-1α and MIP-1β are major suppressive factor released by CD8⁺ T lymphocytes (Cocchi, 1995). The CC chemokines were also shown to suppress infection by M-tropic HIV-1 strains but had little effect on T-cell tropic strain. A chemokine receptor was identified with precisely the corresponding specificity for RANTES, MIP1-α and MIP-1β; it was designated \textit{CCR5} (fifth receptor for CC chemokines) (Deng, \textit{et al.}, 1996; Dragic, \textit{et al.}, 1996; Doranz, \textit{et al.}, 1996).

\textbf{The Expanding Coreceptor Repertoire}

Additional complexity results from the findings that HIV-1 coreceptor activity is not limited to CXCR4 and CCR5. Studies with recombinant proteins demonstrated coreceptor activity for several other human chemokine receptors and related orphans. These include the chemokine receptors CCR2b (Doranz, \textit{et al.}, 1996), CCR3 (Choe, \textit{et al.}, 1996; Doranz, \textit{et al.}, 1996), CCR8 (Rucker, \textit{et al.}, 1997; Horuk, \textit{et al.}, 1998; Jinno, \textit{et al.}, 1998) and CX3CR1 (Rucker, \textit{et al.}, 1997; Combadiere, \textit{et al.}, 1998); the chemokine receptor like orphans STRL33/BONZO (Liao, \textit{et al.}, 1997; Deng, \textit{et al.}, 1997), GPR15/BOB (Deng, \textit{et al.}, 1997; Farzan, 1997) and Apj (Choe, \textit{et al.}, 1996; Edinger, \textit{et al.}, 1998). Not all members of the human chemokine receptor family can function as HIV-1 coreceptors; absence of activity has been noted in most studies on CCR1, CCR4 and CCR6, for CXC chemokine receptors other than CXCR4 and for most several other chemokine receptor-like orphans. In addition to these human
proteins, HIV-1 coreceptor activity has been detected for US28, a CC chemokine receptor encoded by human cytomegalovirus (Rucker, 1997; Pleskoff, 1997).

**HIV-1 CORECEPTOR- CCR5**

**Structure**

The human CC chemokine receptor CCR5 gene encodes a cell surface receptor of 352 amino acid residues in length. The CCR5 coreceptor has seven transmembrane helices, with an N-terminal domain and three extracellular loops on the cell surface, and the C terminus and three intracellular loops. The C termini of the receptor is rich in conserved serine and threonine residues and represent potential phosphorylation sites by the family of G-protein-coupled receptor kinases following ligand binding. Binding of the ligand to CCR5 is critically determined by a single domain- the second extracellular loop (Wu, 1997). The intracellular domain of the receptor is comprised of three loops and the C terminus, which are involved in transduction of the chemokine-mediated signal.

**Role in HIV entry**

The process of HIV-1 entry can be divided into three sequential steps:

- Attachment of the virus to host cells
- Interaction of the virus with coreceptors
- Fusion of the virus and the host cell membrane.

Briefly, the HIV-1 envelop on the surface of the virions is composed of a trimeric CD4-gp120-gp41 complex. In such a complex, the gp41 fusion peptide is buried. The binding of the glycoprotein gp120 to CD4 results in conformational changes (Wyatt and Sodroski, 1998) that induce exposure of the gp120 V3 loop that subsequently interacts with structural elements of the coreceptor (In case of CCR5 the second extracellular domain interacts with the V3 loop). The interaction of gp120 with the coreceptor triggers Env to undergo another conformational change, leading to extension of gp41 and insertion of the fusion peptide into the target cell membrane (Chan, et al., 1998; Doms, et al., 1997).
MEMBRANE TOPOLOGY OF HIV-1 CORECEPTOR CCR5
MODEL OF HIV-1 INDUCED CELL FUSION

Gp120–CD4 and CCR5 binding

Fusion peptide exposure and rearrangement

Fusion peptide intramolecular interactions and membrane fusion
Role of coreceptors in HIV transmission and progression

The realization that CCR5 is the molecular factor mediating entry of the preferentially transmitted M tropic HIV-1 variants led to a focus on this coreceptor as a possible determinant of transmission. Definitive evidence came from the discovery of a mutant CCR5 allele designated CCR5 Δ32 and the association of this allele with resistance to HIV-1 infection (Huang, 1996; Michael, 1997; Hussain, et al., 1998). CCR5 Δ32 has a 32 base pair deletion in the region of the open reading frame encoding the second extracellular loop, causing a frameshift and premature stop codon in the transmembrane domain 5. The truncated protein product is not expressed on the cell surface. It was seen that individuals carrying homozygous CCR5 Δ32 are resistant to HIV infection whereas heterozygotes show delayed disease progression by causing reduced viral replication through reduced expression of CCR5.

Other coreceptor/chemokine genetic polymorphism has also been identified and correlated with delayed HIV-1 disease progression rate, these are CCR5 59029 G/A (Mcdermott, et al., 1998), CCR2-64I (Kostrikis, et al., 1998), and SDF-1 3′UTR-801G-A (or SDF-1 3′A) (Winkler, et al., 1998).

CORECEPTOR- BASED THERAPEUTIC STRATEGIES

HIV coreceptors as antiviral targets:

Based on the current level of understanding, it is anticipated that antiviral therapies that target the CCR5 or CXCR4 receptors would be effective at different stages of disease in an infected individual. Antivirals targeting CCR5 would be most effective for early intervention therapies, whereas antivirals targeting CXCR4 should provide more effective therapy for patients in the later stages of AIDS.

Coreceptor Blocking Agents: Derivatized chemokine variants and chemokine based synthetic peptides have been reported for HIV-1 therapy by blocking of fusion/entry/infection mediated by corresponding coreceptors. Anti-coreceptor monoclonal antibodies that inhibit HIV-1 entry represent another class of
blocking agent. Certain low molecular weight compounds bind directly to the coreceptor and inhibit their function.

**Ex vivo Modulation of Coreceptor Expression:** This therapy involves ex vivo activation of CD4+ T lymphocytes with antibodies to CD3 and CD28 absorbed on beads resulting in a population of CD4+ memory cells that have down modulated transcription of CCR5 and produce factors that inhibit R5 as well as X4 virus replication.

**Gene Therapy Approaches:** Several strategies are suggested in the context of gene therapy, with the goal of depleting coreceptors from the surface of the target cells.

**Intrakines:** This strategy involves the expression of a so-called “intrakine”, a genetically engineered chemokine with a carboxy-terminal endoplasmic reticulum retention sequence; the intrakine traps the newly synthesized coreceptor and prevents its expression on the surface, thereby rendering the target cell refractory to HIV-1 infection. Intrakines have been described for downregulation of CXCR4 (Chen, *et al.*, 1997) and CCR5 (Yang, *et al.*, 1997).

**Intrabodies:** are intracellular antibodies that bind to HIV-1 encoded proteins intracelluarly and prevent their function and/or incorporation into virions (Chen, *et al.*, 1996; Inouye, *et al.*, 1997). This strategy is now being used to trap chemokine receptors using specific mAbs.

**Ribozymes:** are enzymatic RNA molecules that can be designed to specifically cleave other RNAs. Ribozyme targeting sequences can be modified to recognise many accessible target sites within target RNA sequences. Ribozymes have been developed to target CCR5 and CXCR4 coreceptor RNA. Inhibition of CCR5 dependent HIV-1 infection by hairpin ribozyme has been shown recently (Feng, *et al.*, 2000).
CHEMOKINES, CORECEPTORS AND HIV LIFE CYCLE

Steps at which HIV/chemokine receptor interaction can be disrupted, include:
(1) Downregulation of chemokine receptor with anti CD3 and CD28 mAbs;
(2) site for ribozyme action;
(3) and (4) newly synthesized CCR5 and CXCR4 form complexes with intrakines and intrabodies;
(5) extracellular mAbs to CCR5 block;
(6) suicide viruses target gp120/41 expressing cells; and
(7) chemokines, peptides, small molecules bind to their cognate receptors and prevent interaction with gp120/41.
A summary of the status of anti-HIV-1 therapeutic strategies involving chemokine receptors is given below:

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Therapy</th>
<th>Target</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune restoration</td>
<td>Downregulation of CCR5 on CD4+ T cells</td>
<td>CCR5</td>
<td>Phase I human trials</td>
</tr>
<tr>
<td></td>
<td>Use of -/- CCR5 cells</td>
<td>CCR5</td>
<td>concept</td>
</tr>
<tr>
<td>Gene Therapy</td>
<td>Ribozymes</td>
<td>CCR5, CXCR4</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>Intrakines</td>
<td>CCR5, CXCR4</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>Single chain mAbs</td>
<td>CCR5, CXCR4</td>
<td>concept</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CCR5, CXCR4</td>
<td>concept</td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>mAbs</td>
<td>CCR5, CXCR4</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Suicide vectors</td>
<td>Modified cytopathic viruses or vectors</td>
<td>HIV-1 infected cells</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Chemokines/peptides</td>
<td>MIP1-α</td>
<td>CCR5</td>
<td>Phase I human trials</td>
</tr>
<tr>
<td></td>
<td>Met-RANTES</td>
<td>CCR5</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>AOP-RANTES</td>
<td>CCR5</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>T22</td>
<td>CXCR4</td>
<td>Preclinical</td>
</tr>
<tr>
<td></td>
<td>ALX40-4C</td>
<td>CXCR4</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Small molecule antagonists</td>
<td>AMD310</td>
<td>CXCR4</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>
The work done in the present study is focussed on the following objectives:

1. Efficacy of mono and multitarget -ribozymes against the X gene of Hepatitis B virus

2. Designing and study of efficacy of mono and Di-DNA-enzymes against Hepatitis B virus X gene.

3. Studying cleavage activity of a mono-ribozyme and a mono-DNA-enzyme against the HIV-1 coreceptor CCR5 gene.